[CONTRIBUTION FROM THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

The Reactions of Antiserum Homologous to the *p*-Azohippurate Ion[1]

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A study has been made of the combination of ions structurally related to hippurate ion with antibodies prepared against *p*-azohippurate ion. The inhibitory action of these ions on the precipitation of antibody with antigen has been measured quantitatively, and the results expressed in terms of the relative free energy change in the combination of these ions with antibody. The antibody was found to fit very closely around the glycine residue of the hippurate ion. Replacement of an α-hydrogen by an alkyl group decreases the free energy of combination with antibody by more than 2300 cal. Replacement of the NH group of hippurate by a methylene group decreases the free energy of combination by 1100 cal. Replacement of the benzoyl group by an acetyl, benzenesulfonyl or phenylcarbamyl group results in a large decrease in the free energy of combination, as does any variation in the carbonyl–carboxylate distance. Substitution of a nitro group on the benzene ring of hippurate ion increases the free energy of combination due to the large van der Waals interaction of the nitro group. Malonanilate ion is nearly as effective as hippurate ion itself in combining with antibody. This similarity in action may be explained by a steric similarity in the hydrated malonanilate and hippurate ions.

In the study of the specificities of antibodies prepared against simple substances the observation has been made repeatedly that the replacement of a methylene group in a hapten by the imino NH group reduces its combination with antibody which has been prepared against the substance containing the methylene group. Thus, we have found that phenylhydantoate ion does not combine as well as does succinanilate with antibody prepared against the latter ion,[2] and also that hippurate does not combine as well as benzoylpropionate with antibodies prepared against benzoylpropionate.[3] The differences in combining strength have been greater than would have been expected from the difference in van der Waals interaction or from the difference in size of the CH_2 and NH groups. The difference in combining strength has been attributed to the hydration of the NH group which would give it an appreciably greater size and a consequent steric hindrance to the combination. In order to investigate the combining properties and the specificities of antibodies prepared against compounds with the NH grouping we have made a study of antibodies against the *p*-azohippurate ion. Antibodies against the *p*-azohippurate ion were first studied by van der Scheer and Landsteiner,[4] who determined their reactions with the *p*-nitrobenzoyl derivatives of several amino acids and peptides.

Experimental Methods

Materials.—With the exceptions of the preparations described below, all the simple haptens used in these studies have been described previously[2] or were commercial preparations crystallized to the correct melting point and neutral equivalent. Normal beef and normal rabbit sera were regenerated preparations of material stored in the lyophilized form. Crystallized ovalbumin was obtained from Armour and Co.

The benzoylated amino acids were prepared by the Schotten–Baumann reaction of the *dl*-amino acids with benzoyl chloride. **Acetylglycine** was prepared by a similar procedure with acetic anhydride. **Benzenesulfonylglycine** was obtained by vigorously stirring for 3 hours a solution of 2 g. of benzenesulfonyl chloride in 25 ml. of ether with 2 g. of glycine in 20 ml. of 1 *N* sodium hydroxide. The product was isolated on acidification of the aqueous layer with hydrochloric acid. ***p*-Aminohippuric acid** was prepared by catalytically reducing (5% Pd on $BaSO_4$) at an initial hydrogen pressure of 42 lb./sq. inch an ethanolic solution of *p*-nitrohippuric acid. The calculated quantity of hydrogen was adsorbed in 3 minutes. Catalyst was separated by filtration and the product isolated by concentration *in vacuo*. The melting points and recrystallization solvents for the amino acid derivative are: ***dl*-benzoylphenylalanine** (40% EtOH, 184–185°); ***dl*-α-benzoylaminovaleric acid** (40% EtOH, 150.5–152.5°); ***dl*-benzoylalanine** (H_2O, 165–166.5°); ***dl*-α-benzoylaminocaproic acid** (20% EtOH, 134.5–136°); **acetylglycine** (50% EtOH, 207–208°); **benzenesulfonylglycine** (H_2O, 166.5–168°); ***p*-aminohippuric acid** (H_2O, 196–196.5°); ***dl*-α-benzoylaminobutyric acid** (H_2O, 143–145°).

Protein Antigens.—The antigen used for injection in the rabbits was prepared by diazotizing 170 mg. of *p*-aminohippuric acid and coupling at 4° with 35 ml. of beef serum (4 g. of lyophilized material) to which 10 ml. of 5% sodium carbonate was added. The azoprotein was dialyzed for 4 days *vs.* many changes of saline. The test antigen was prepared by diazotizing 123 mg. of *p*-aminohippuric acid and coupling with 500 mg. of ovalbumin at *p*H 9.5. The solution stood overnight at 3–5° and the azoprotein was purified by dialyzing for 24 hours *vs.* saline borate, twice precipitating at *p*H 3.6, four times washing with cold 60% acetone and finally dissolving in 50 ml. of saline at *p*H 8.

Preparation of Antisera.—Antisera were obtained and pooled in a manner similar to that described for the preparation of anti-R_p sera.[5]

Reaction of Antiserum with Antigen and Hapten.—The reactants were mixed and allowed to stand for 3 days at 5°. The precipitates were centrifuged, washed 3 times with 8-ml. portions of cold 0.16 *M* solutions of sodium chloride, and analyzed by a modified Folin procedure.[6]

Albumin Binding.—The binding of several haptens to a threefold borate buffer[7] dilution of normal rabbit serum at 5° was determined by a method of equilibrium dialysis.[8]

Results

The Extent of Combination of Hapten with Antibody.—The extent of combination of hapten with antibody was determined by the ability of the hapten to inhibit the precipitation of the anti-hippurate antibody with hippurate–ovalbumin. The amount of antigen used was that which gave the optimum amount of precipitate with antibody at *p*H 8. As in other antibody–antigen systems the antiserum gave the largest amount of precipitate with an optimum amount of antigen and less with either more or less antigen. The *p*H was kept at 8 since the carboxylic acids would be essentially completely dissociated, and since other

(1) This research was jointly supported by the Office of Naval Research and the U. S. Atomic Energy Commission.

(2) D. Pressman, J. H. Brdyen and L. Pauling, THIS JOURNAL, **70**, 1352 (1948).

(3) D. Pressman and M. Siegel, *ibid.*, **75**, 1376 (1953).

(4) J. van der Scheer and K. Landsteiner, *J. Immunol.*, **29**, 171 (1935).

(5) L. Pauling, D. Pressman, D. Campbell, C. Ikeda and M. Ikawa, THIS JOURNAL, **64**, 2994 (1942).

(6) D. Pressman, *Ind. Eng. Chem., Anal. Ed.*, **51**, 337 (1943).

(7) L. Pauling, D. Pressman, D. H. Campbell and C. Ikeda, THIS JOURNAL, **64**, 3163 (1942).

(8) D. Pressman and M. Siegel, *Arch. Biochem.*, in press.

TABLE I

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-HIPPURATE SERUM WITH HIPPURATE–OVALBUMIN

Anti-H_p serum, 0.50 ml.; H_p-ovalbumin in borate buffer, 0.50 ml. (67 μg. protein); hapten in saline, 0.50 ml., 90 minutes at 37° and three days at 5°

Hapten		K'_0	σ	ΔF_{rel}	Hapten concentration, molar × 10^5 5.2	10.4	20.8	41.7	83.3	167	333	667	1330	2670
					Amount of precipitate[a]									
Series A														
Hippurate	$C_6H_5CNHCH_2COO^-$	1.00	2	0		690		410		80				
p-Nitrohippurate	*p*-$NO_2C_6H_4CNHCH_2COO^-$	1.4	1.5	−190	840		510		300					
β-Benzoylpropionate	$C_6H_5CCH_2CH_2COO^-$	0.14	3	1100				690		460		270		
Succinanilate	$C_6H_5NHCCH_2CH_2COO^-$	.030	2.5	1900						730		480		320
Phenylhydantoate	$C_6H_5NHCNHCH_2COO^-$	.085	2.5	1400					690		470		240	
Levulinate	$CH_3CCH_2CH_2COO^-$	.0040	1.5	3100						1000		890		690
γ-Benzoylbutyrate	$C_6H_5CCH_2CH_2CH_2COO^-$	.024	2.5	2100						780		500		340
γ-Phenylbutyrate	$C_6H_5CH_2CH_2CH_2COO^-$	.0085	3	2600						850		680		450
Malonanilate	$C_6H_5NHCCH_2COO^-$	.25	1.5	770			880		540		300			
Series B[b]														
Hippurate	$C_6H_5CNHCH_2COO^-$	1.00	1.5	0		850		500		150				
N-Benzoylalanate	$C_6H_5CNHCHCOO^-$ (CH₃)	0.016	3	2300					900		770		570	
α-N-Benzoylaminobutyrate	$C_6H_5CNHCHCOO^-$ (C_2H_5)	.006	0	2800						980		910		820
α-N-Benzoylaminovalerate	$C_6H_5CNHCHCOO^-$ (C_3H_7)	<.006		>2800						970		920		900
α-N-Benzoylaminocaproate	$C_6H_5CNHCHCOO^-$ (C_4H_9)	<.006		>2800						950		930		850
N-Benzoylphenylalanate	$C_6H_5CNHCHCOO^-$ ($CH_2C_6H_5$)	.007	1	2700						970		900		700
N-Acetylglycinate	$CH_3CNHCH_2COO^-$	.012	0	2400						970		860		640
Benzenesulfonylglycinate	$C_6H_5SNHCH_2COO^-$ (O)	.013	1	2400						950		860		530

[a] The amount of precipitate is reported in parts per mille of the amount present in the absence of hapten, 252 μg. These values are averages of triplicate analyses with mean deviation of 3%. [b] The haptens of Series B were run with the same volume of a different pool of Anti-H_p Serum and with a different concentration of H_p-ovalbumin (201 μg. protein). The amount of precipitate in the absence of hapten was 717 μg. The averages of triplicate analyses have a mean deviation of 3%.

systems have been investigated under the same conditions. Data on the effect of hapten on the amount of precipitate obtained are given in Table I. Values of the hapten inhibition constant K'_0 and the heterogeneity index σ, obtained on application of the theory of heterogeneous antisera,[9] are listed. There are also listed values for the relative free energy of combination of antibody with hapten. These values are relative to the values of combination of antibody with the homologous hippurate hapten. The haptens were run in two groups with different pools of sera, and are labeled series A and B in Table I. Since these determinations were carried out in the presence of whole serum, some correction was required to compensate for the binding of certain of the haptens by the serum albumin present. This correction may be large at very low hapten concentrations, but is vanishingly small at high hapten concentrations. The measured extent of binding to normal rabbit serum (threefold dilution with borate buffer) of those haptens which exhibited inhibition at low concentrations is reported in Table II together with the fiducial concentration[9] and the per cent. bound at the fiducial concentration. The K'_0 values in Table I are calculated from the free concentration of hapten in the antibody–antigen–hapten system, *i.e.*, the total concentration of hapten minus the concentration bound to albumin.

(9) L. Pauling, D. Pressman and A. Grossberg, THIS JOURNAL, **66**, 784 (1944).

Discussion

The substitution of a nitro group into the benzene ring in the para position (the position at which the hippurate ion was attached to the immunizing protein antigen) decreased the relative free energy of combination by 200 cal. This is due to the greater van der Waals interaction of the antibody with the para nitro group than with the hydrogen

TABLE II

BINDING OF SIMPLE HAPTENS TO NORMAL RABBIT SERUM (THREEFOLD BORATE BUFFER DILUTION) AT 4°

Hapten	Concn. in protein phase, M	% bound[a]	Fiducial concn., M	% bound[b] at fid. concn.
Hippurate	1.3×10^{-4}	54 ± 0	2.8×10^{-4}	35
	3.5×10^{-4}	28 ± 2	4.6×10^{-4}	26
	1.9×10^{-3}	16 ± 0		
p-Nitrohippurate	1.6×10^{-4}	48 ± 1	2.3×10^{-4}	44
	3.1×10^{-4}	40 ± 3		
β-Benzoylpropionate	7.4×10^{-4}	37 ± 2	1.7×10^{-3}	23
	1.2×10^{-3}	30 ± 2		
Phenylhydantoate	2.2×10^{-3}	18 ± 1	2.6×10^{-3}	16
	3.2×10^{-3}	14 ± 1		
Malonanilate	7.2×10^{-4}	32 ± 2	1.0×10^{-3}	29
	1.2×10^{-3}	27 ± 4		

[a] Reported as average of duplicate determinations. [b] Calculated by linear interpolation.

of the unsubstituted compound. The substitution of a nitro group in the para position of benzoate or benzenearsonate ion decreased the free energy of combination of these haptens with their homologous antibodies to the extent of 800 cal. or more.[10,11] The greater interaction with antibodies prepared against these compounds indicates a close enough fit of antibody around the substituent in the para position of the benzene ring to allow the greater van der Waals interaction to come into play.[12] In the case of the hippurate ion the 200 cal. interaction due to the nitro group indicates a relatively loose fit of antibody around the azo group and benzene ring of the immunizing hapten.

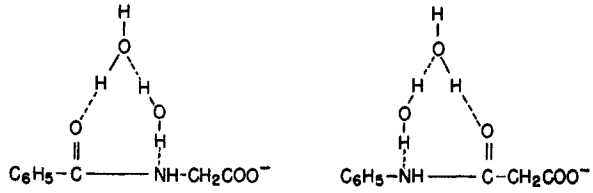

Fig. 1.—Structure of hydrated hippurate and malonanilate ions.

The fit of antibody around the glycine rest of the hippurate is very close. The replacement by a methyl group of a hydrogen on the carbon atom α to the carboxylate ion (as in N-benzoylalanate) causes a large decrease in combining ability (2300 cal. increase in ΔF). Increasing the size of the substituent to ethyl, propyl, butyl or benzyl, as in α-N-benzoylaminobutyrate, α-N-benzoylaminovalerate, α-N-benzoylaminocaproate and N-benzoylphenylalanate, increases ΔF still further.

Replacement of the NH group of hippurate by a methylene group to give β-benzoylpropionate ion also results in a decrease in combining ability. The corresponding increase in the free energy of combination, 1100 cal., is a larger change than is found on the substitution of methylene CH_2 for imino NH in going from phenylhydantoate to succinanilate or from N-acetylglycinate to levulinate, 500 and 700 cal., respectively. These lower values probably reflect the decreased combining power already present in the two molecules containing aniline or methyl groups rather than the benzene ring. The greater combining power of the compounds containing the NH group on the α carbon atom, may be due to the fact that the NH group is hydrated and thus quite different sterically from the CH_2 group. This is especially reasonable if antibody specificity is directed against the hydrated NH group. It is also possible that the antibody has a proton donor or acceptor group in the region where it can complex with the NH group increasing the strength of combination.

The fit of antibody around the carbonyl group is too close to accommodate a sulfonyl group in place of the carbonyl. The increase in ΔF of 2400 cal. for benzenesulfonylglycinate is presumably due to the greater size of the sulfonyl group.

Displacement of the carbonyl group one carbon atom from its homologous position (γ-benzoylbutyrate) does not completely destroy its combining ability. Although ΔF_{rel} is increased by 1000 cal. over that of the β-benzoylpropionate, it is interesting that it still combines more strongly (500 cal.) than γ-phenylbutyrate. The complete loss of the hydrogen bonding properties by the removal of the carbonyl oxygen is more important than increasing the distance between the benzoyl and carboxylate group.

The replacement of the benzene grouping by an anilino grouping increases the free energy of combination by 1400 cal. in going from hippurate to phenylhydantoate, or 800 cal. in going from β-benzoylpropionate to succinanilate. (It is consistently observed that a structural variation applied to the homologous ion has a greater effect than the same variation applied to an ion differing from the homologous ion in some other respect. In the above case the β-benzoylpropionate differs from the ion against which the antiserum was prepared by the substitution of a methylene for the amino group). This increase in energy of combination is presumably due to the increase in the benzene–carboxylate distance. If the antibody fits closely around both the benzene ring and the carboxylate of the immunizing hapten, then altering this distance in other haptens will result in their poor combination. The importance of the van der Waals contribution of the benzene ring is indicated

(10) D. Pressman, S. Swingle, A. Grossberg and L. Pauling, THIS JOURNAL, **66**, 1731 (1944).

(11) L. Pauling and D. Pressman, *ibid.*, **67**, 1003 (1945).

(12) The van der Waals attraction varies inversely with the sixth power of the distance between groups and thus drops off rapidly with separation due to poor fit. (See ref. 11).

by replacing it with a methyl group (acetylglycinate). This increases ΔF by 2400 cal.

It is interesting that malonanilate ion in which the NH and carbonyl groups are interchanged has a relatively high combining constant, within 500 cal. of that of hippurate itself. This may be due to the possibility that both the NH and carbonyl groups are hydrated and the compound with the hydrated NH and carbonyl groups reversed (Fig. 1) appears structurally and configurationally quite like the original hydrated hippurate as far as the antihippurate antibodies are concerned. This is especially apparent upon examination of a three dimensional (to scale) molecular model.

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An Agent from *E. Coli* Causing Hemorrhage and Regression of an Experimental Mouse Tumor. IV. Some Nitrogenous Components of the Phospholipide Moiety[1]

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D-Glucosamine, ethanolamine and a hitherto unreported diamine have been found to be components of the phospholipide moiety of the mouse tumor hemorrhagic agent. The diamine, for which the name *necrosamine* is proposed, has been assigned the provisional formula $CH_3(CH_2)_{14}CH(NH_2)CH(NH_2)C_3H_7$. There is some evidence that aspartic acid may also be a component of the phospholipide moiety.

The agent which is elaborated by *E. coli* and which produces a hemorrhagic response in and causes the regression of the experimental mouse sarcoma 180 is a complex polysaccharide containing both a peptide and a phospholipide moiety.[3] It has been shown previously,[4,5] that the polysaccharide is composed of D-glucose, D-galactose and D-glucosamine, with the latter probably present as N-acetyl-D-glucosamine,[3] and that the fatty acids present in the phospholipide moiety are lauric, myristic, palmitic and D-β-hydroxymyristic acid. The phospholipide moiety[3,5] upon acid hydrolysis gave, in addition to the above ether-soluble fatty acids, a water-soluble fraction and a flocculent precipitate which was insoluble both in water and in ether.[5] It is the purpose of this communication to report on the nature of this latter substance and upon some of the other nitrogenous components of the water soluble fraction.

Treatment of the flocculent precipitate, which was obtained in *ca.* 12% yield from the phospholipide,[5] with aqueous sodium hydroxide followed by solution in ether and subsequent precipitation with methanolic hydrogen chloride gave, in good yield, a crystalline compound which appeared to be the hydrochloride of an amine. Elementary analysis of the hydrochloride, the picrate and the benzoyl derivative of this amine, and a molecular weight determination of the latter derivative, indicated that the compound in question was an acyclic saturated diamine with the molecular formula $C_{20}H_{44}N_2$. A Kuhn–Roth determination indicated the presence of at least two terminal methyl groups and thus suggested the probability of finding the amino groups in non-terminal positions. Since the diamine was observed to react with carbon disulfide to form an intramolecular dithiocarbamate salt[6] which, upon heating, lost hydrogen sulfide to form a cyclic thiourea, it was presumed that the two amino groups were in contiguous or near contiguous positions.

As the diamine under investigation appeared to be rather slowly attacked by lead tetraacetate at 25° all subsequent studies with this reagent were conducted at 60°, *cf.* Table I. Under these conditions, *i.e.*, at 60° for one hour, both ethylene glycol and 2,3-butylene glycol still consumed only one mole of the reagent per mole of substrate. However, under the same conditions, 4.4 moles of reagent was consumed per mole of diamine. It is clear from the data given in Table I that this result is not an unreasonable one for a compound of the type $-CH_2-CHNH_2-CHNH_2-CH_2-$ since lead tetraacetate, under the conditions previously speci-

TABLE I

REACTION OF LEAD TETRAACETATE WITH CERTAIN NITROGENOUS COMPOUNDS AND GLYCOLS[a]

Type	Compound	Moles $Pb(OAc)_4$ / Mole comp.
1,2-Glycols	Ethylene glycol	1.0
	2,3-Butylene glycol	1.0
Monoamines	*s*-Butylamine	0.3
	n-Decylamine	0.4
1,2-Hydroxyamines	Ethanolamine	1.5
	DL-Threonine	3.7
1,2-Diamines	Ethylenediamine	1.7
	DL-2,3-Diaminobutane	3.7
	Necrosamine	4.4
1,3-Diamines	2,4-Diaminopentane	1.2
Nitriles	Acetonitrile	0.5
	Capronitrile	0.3

[a] In glacial acetic acid for one hour at 60°.

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(2) To whom inquiries regarding this article should be sent.

(3) M. Ikawa, J. B. Koepfli, S. G. Mudd and C. Niemann, *J. Nat. Cancer Inst.*, **13**, 157 (1952).

(4) M. Ikawa, J. B. Koepfli, S. G. Mudd and C. Niemann, THIS JOURNAL, **74**, 5219 (1952).

(5) M. Ikawa, J. B. Koepfli, S. G. Mudd and C. Niemann, *ibid.*, **75**, 1035 (1953).

(6) E. R. Buchman, A. O. Reims, T. Skei and M. J. Schlatter, *ibid.*, **64**, 2696 (1942).